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TECHNICAL MANUSCRIPT 186

β-PHENETHYL ALCOHOL INHIBITION OF BACILLUS SUBTILIS

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β-PHENETHYL ALCOHOL INHIBITION OF BACILLUS SUBTILIS

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ABSTRACT

Initial studies of β-phenethyl alcohol (PEA) inhibition in Bacillus subtilis strain 168 indole⁻ reveal that concentrations necessary to inhibit growth fall within very narrow limits. Under our conditions stationary phase cells of strain 168 could not undergo inhibition without concomitant loss of viability. Log-phase cells were capable of survival in PEA but unable to resume log-phase growth when removed from PEA.

Strain W23 responded differently in that inhibited cells were found capable of recovery when removed from the inhibitor. Cells of different stages of growth exhibited differences in response to PEA inhibition and to relief from PEA inhibition.

Chemical studies of inhibited W23 cells show a rapid cessation of DNA, RNA, and protein synthesis. Cells recovering from inhibition were found to lag 1 hour prior to initiation of synthesis. No DNA synthesis was detected throughout the 2-hour period of recovery examined.

I. INTRODUCTION

β-Phenethyl alcohol (PEA) has been shown to effect a dissociation of macromolecular synthesis in Escherichia coli.[1] Cells in the ideal physiological condition under PEA inhibition continue to synthesize RNA and proteins but do not synthesize DNA. These cells do not lose colony-forming ability and the entire population can recover (resume DNA synthesis and initiate growth) if the inhibitor is removed. Most recently it has been suggested that PEA suppresses DNA synthesis by preventing the initiation of new rounds of DNA replication.[2]

In Bacillus subtilis, polarity of DNA synthesis has been well documented.[3-6] PEA inhibition and release from PEA inhibition could prove to be useful in studies of the B. subtilis chromosome. Initial studies of PEA inhibition in B. subtilis constitute the subject of this report.

II. MATERIALS AND METHODS

B. subtilis strain 168 indole$^-$ and strain W23 methionine$^-$ were used throughout the studies. Cells were grown in Davis minimal media containing 1% glucose as a carbon source and supplemented with 60 μg/ml indole for strain 168 or 60 μg/ml methionine for strain W23. Twenty-ml cultures were grown in 250-ml Erlenmeyer flasks. These flasks are equipped with a Klett tube fused on top to permit turbidimetric determinations. All cultures were incubated at 37 C on a reciprocal shaker. Klett readings (blue filter) and viability samples were taken as indicated within each experiment. PEA was removed in recovery studies by washing the cells on membrane filters. Standard plating techniques were used.

Perchloric acid extracts were prepared for DNA and RNA determinations. Low-molecular-weight materials were first extracted with cold 0.25 N $HClO_4$. High-molecular-weight nucleic acids were then extracted with 0.5 N $HClO_4$ at 70 C. (The latter extracts were used for DNA and RNA determinations.) DNA was determined by the diphenylamine reaction, RNA by the orcinol method. Proteins were measured by the Lowry method. (The sensitivity of these methods would not detect turnover or small amounts of residual synthesis.)

III. RESULTS

Initial studies of strain 168 were aimed at determining the critical concentration of PEA necessary to stop macromolecular synthesis. Stationary phase cells (overnight at 37 C culture) were used as starting material in light of Yoshikawa and Sueoka's report[4] that a majority of the cells in a stationary phase population contain completed chromosomes. The family of growth curves shown in Fig. 1 were generated by resuspending the pellet obtained from 5-ml samples of an overnight culture in 20 ml of fresh growth media containing the indicated percentages (v/v) of PEA. These cultures were incubated at 37 C with shaking. Turbidimetric (Klett) readings and samples for viability were taken at the times indicated. From Fig. 1 it is evident that the critical concentration of PEA falls within very narrow limits; 0.25% prevents any growth, but 0.24% only delays the onset of growth. Fig. 2 presents viability data corresponding to the turbidity data shown in Fig. 1. Again, a family of curves is shown. The spectrum of slopes is far greater in Fig. 2 than in Fig. 1 especially in the range of higher PEA concentrations. It appears that at concentrations of 0.25% and above these cells are rapidly inactivated although they do not appear to lyse. At concentrations just under 0.25% (not shown on these curves) the cells will overcome inhibition and resume log-phase growth (after about a 6-hr lag). At concentrations of 0.20% and lower only a slight decrease in growth rate occurs. In no case are results similar to those obtained with *E. coli*, that is, a slight increase in turbidity coupled with no increase or loss in colony-forming units.

Similar experiments were performed using log-phase cells as starting materials. Fig. 3 shows the turbidity data obtained in these experiments. By comparison with Fig. 1 it is apparent that higher concentrations of PEA are required to inhibit log-phase cells than to inhibit stationary-phase cells. (In both cases the same concentration of cells was challenged in PEA.) The right side of Fig. 3 depicts an experiment attempting to reverse the effects of PEA inhibition. The cells were incubated for 2 hr at 37 C in 0.28% PEA. PEA was then removed from the cells and turbidity and viability were observed for an additional 2 hr. Although the turbidity of the culture remained constant throughout this period, examination of the corresponding viability data (shown on the right of Fig. 4) reveals that the reversed culture does not maintain viability. A comparison of the data shown on the left of Fig. 3 and 4 indicates, that there is good correlation between viability and turbidity data where the growth rates are near control values.

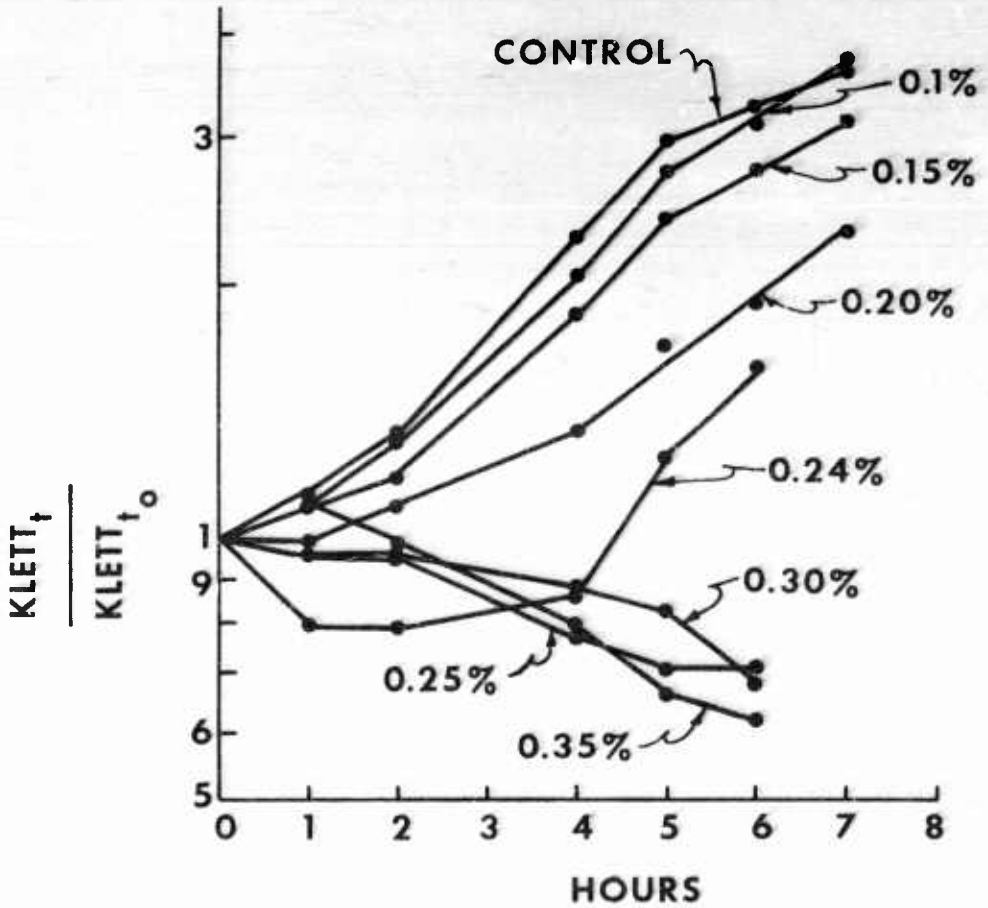

Figure 1. Growth (Turbidity) of B. subtilis Strain 168 Indole$^-$ in β-Phenethyl Alcohol.

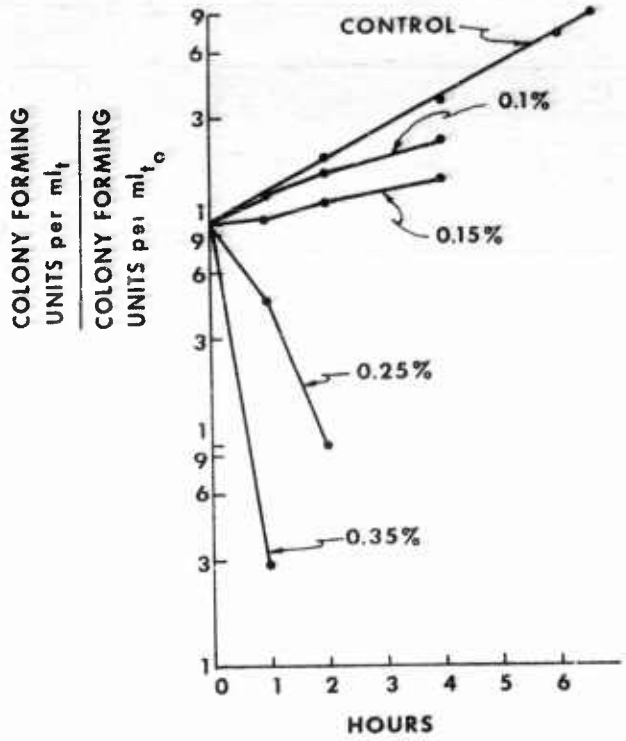

Figure 2. Growth (Viability) of B. subtilis Strain 168 Indole$^-$ in β-Phenethyl Alcohol.

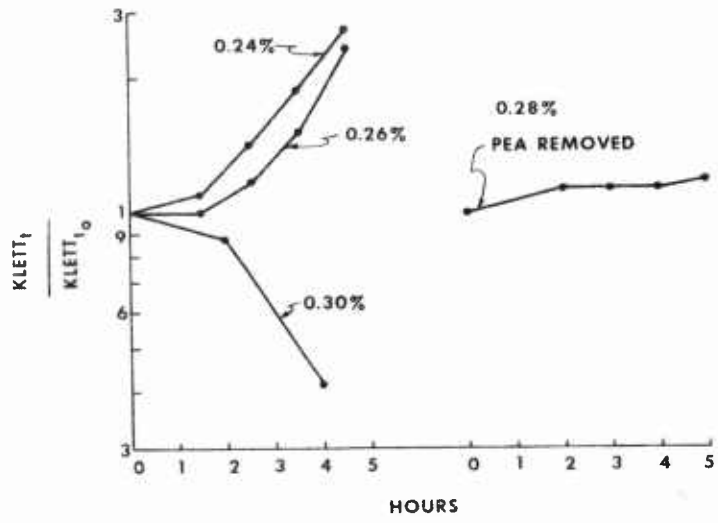

Figure 3. Growth (Turbidity) of B. subtilis Strain 168 Indole$^-$ in β-Phenethyl Alcohol (Log Phase Cells as Starting Material).

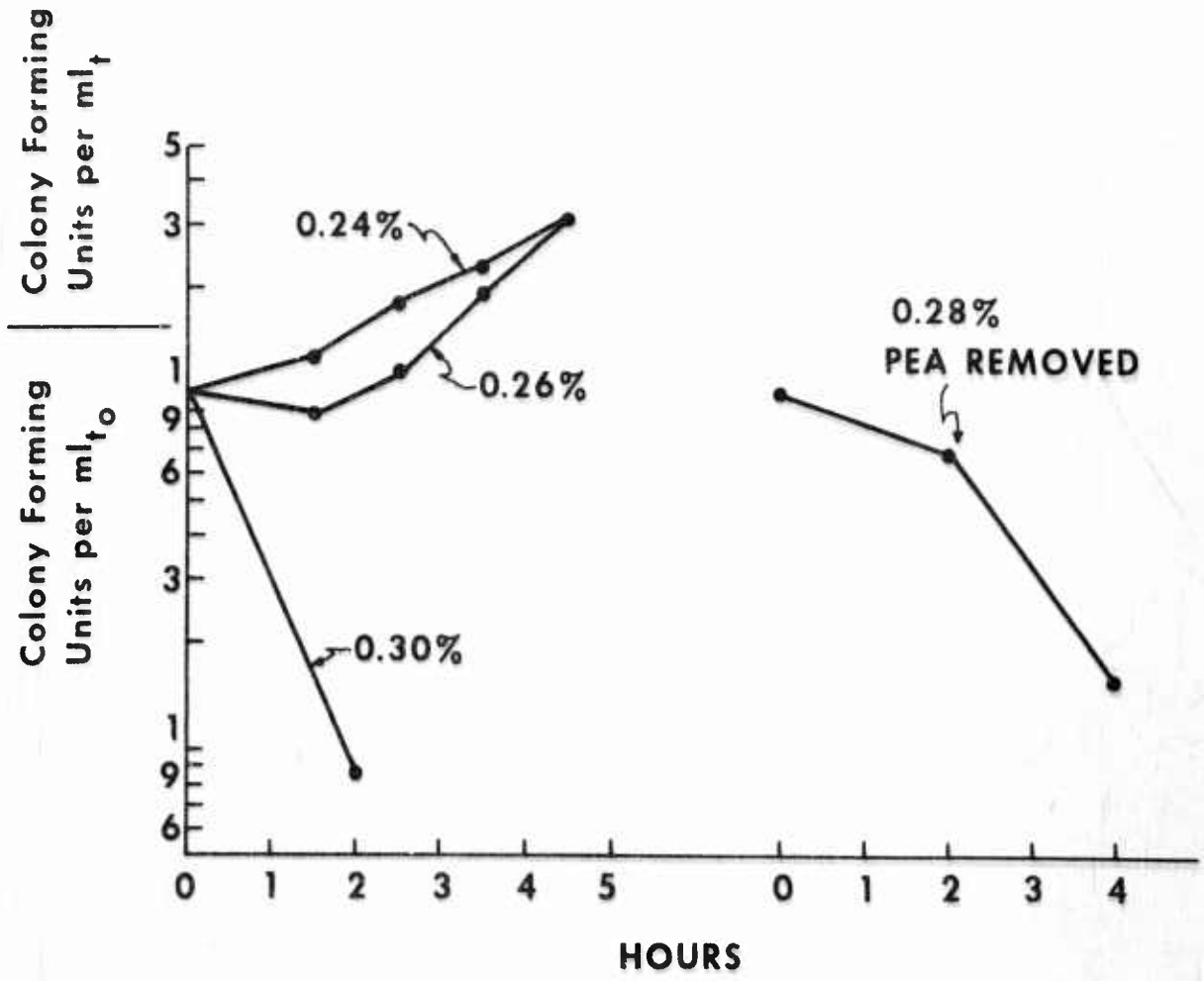

Figure 4. Growth (Viability) of B. subtilis Strain 168 Indole$^-$ in β-Phenethyl Alcohol (Log Phase Cells as Starting Material).

Concurrently with the above studies, strain W23 was studied under similar inhibition conditions. Initial results showed that log-phase cells were inhibited at concentrations similar to those for strain 168 (Fig. 5 and 6). An important difference between the strains was soon noted. Strain W23 showed a striking ability to recover from PEA inhibition. Fig. 7 shows the results of four reversal experiments with 0.29% PEA, using cells at different stages of growth as starting material. Data corresponding to the same experiment are presented in column form. Three parameters are presented for each experiment. The top row of figures shows the turbidity of each culture during inhibition and recovery stages. The center row of figures shows the growth stage (again by turbidity measurement) of the parent culture. The last point on these curves represents the point at which the cells were harvested and resuspended in inhibitor. Finally, the bottom row shows the viability data corresponding to the period of inhibition and recovery.

In the experiment shown in column A an overnight culture was used at starting cells. Therefore, no parent growth stage is shown in the middle row. Column A indicates that there is an initial loss of viability when the cells are resuspended in PEA. There is no loss of turbidity during this period. Upon removal of PEA a rapid increase in both turbidity and colony-forming units is noted. Similar results are seen in columns B, C, and D. In the latter three cases there is no appreciable loss of viability during the time the cells are in PEA. Moreover, one sees an increase in turbidity during this time. This likely represents residual protein synthesis and is analogous to the findings of Berrah and Konetzka,[1] concerning PEA inhibition of E. coli.

DNA, RNA, and protein analysis were performed as described in the methods section. The results of these experiments are shown in Fig. 8, 9, and 10. In the first experiment, stationary-phase cells (W23) were diluted into PEA at the time indicated as 0. No appreciable synthesis is detected over the 2-hr period shown. Although the cells do not lyse during this period, viability decreases appreciably. A sample was removed at 2 hr, washed free of PEA, and resuspended in fresh growth media at 37 C. The progress of this subculture was followed turbidimetrically and is shown on the insert to Fig. 8. The culture was able to recover, although there was a 2-hr lag prior to growth.

Fig. 9 shows the results of the same experiment using log-phase cells as starting material. The cells rapidly stop macromolecular synthesis when confronted with PEA. Although some lysis is evident during the 2-hr incubation period, 80% of the cells retain colony-forming ability throughout this period. More than 90% of the cells survive a 1-hr treatment.

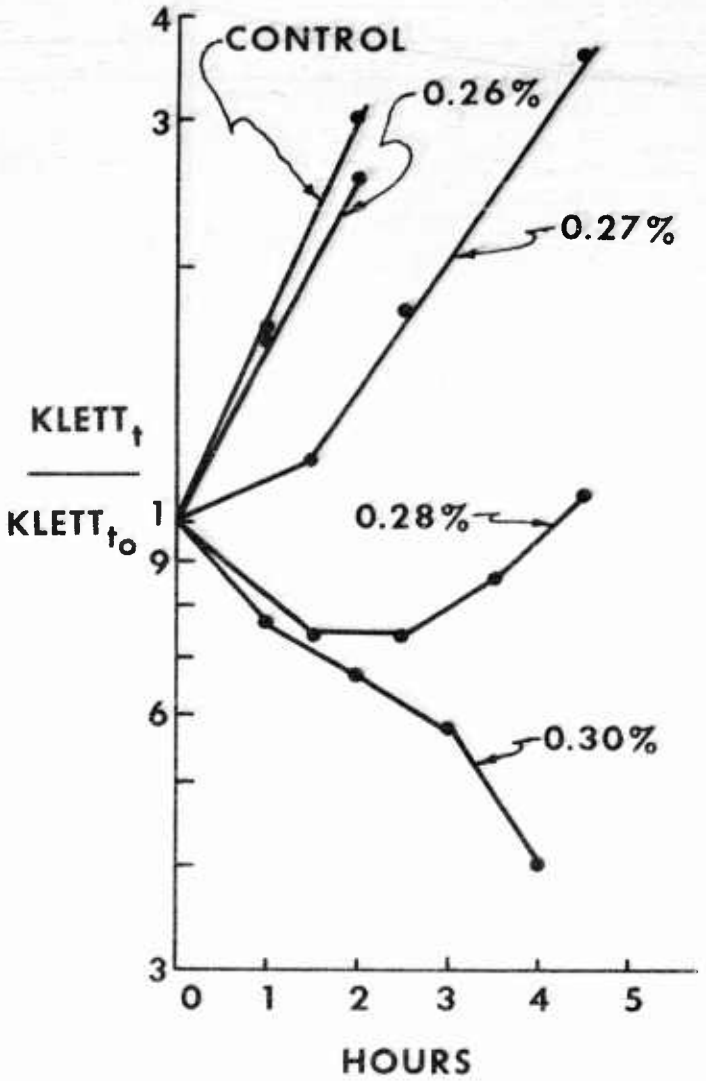

Figure 5. Growth (Turbidity) of B. subtilis Strain W23S^r Methionine$^-$ in β-Phenethyl Alcohol.

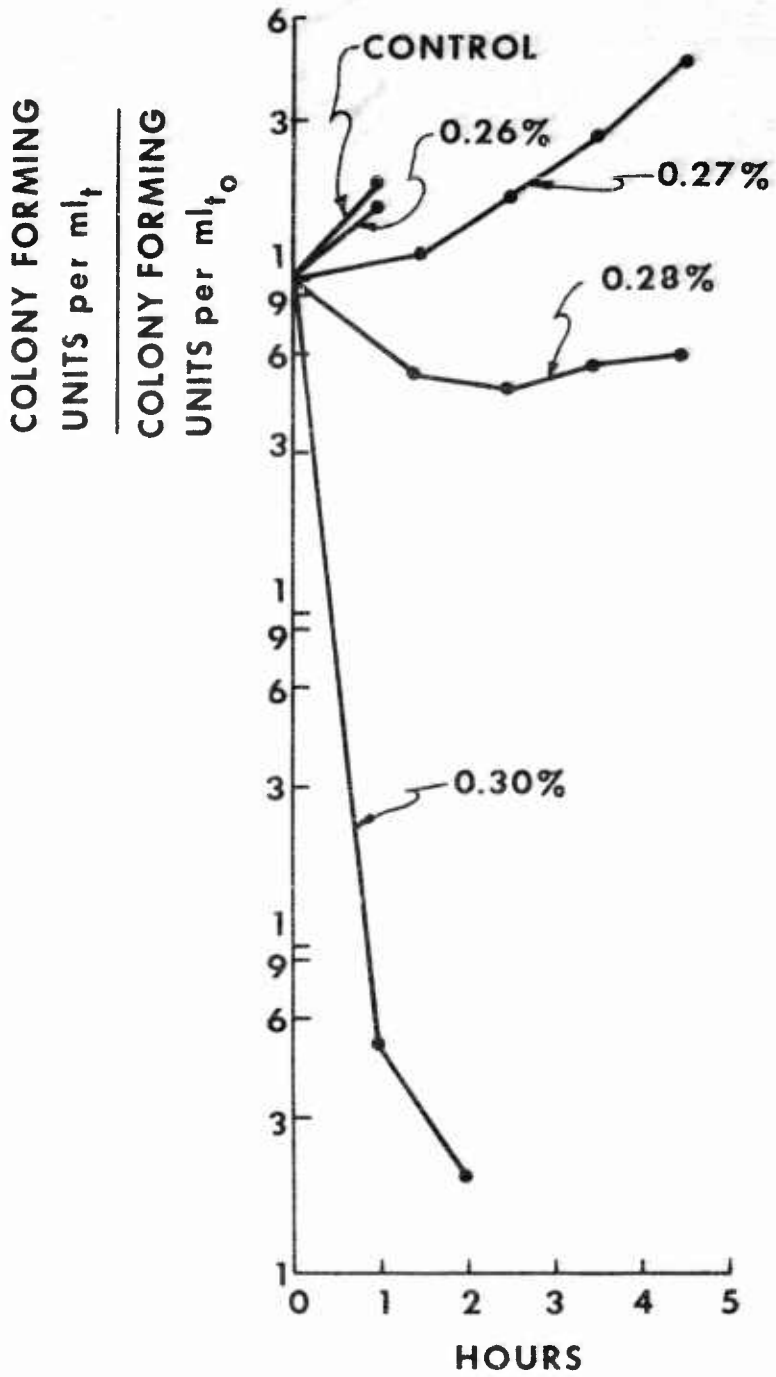

Figure 6. Growth (Viability) of B. subtilis Strain W23S^r Methionine$^-$ in β-Phenethyl Alcohol.

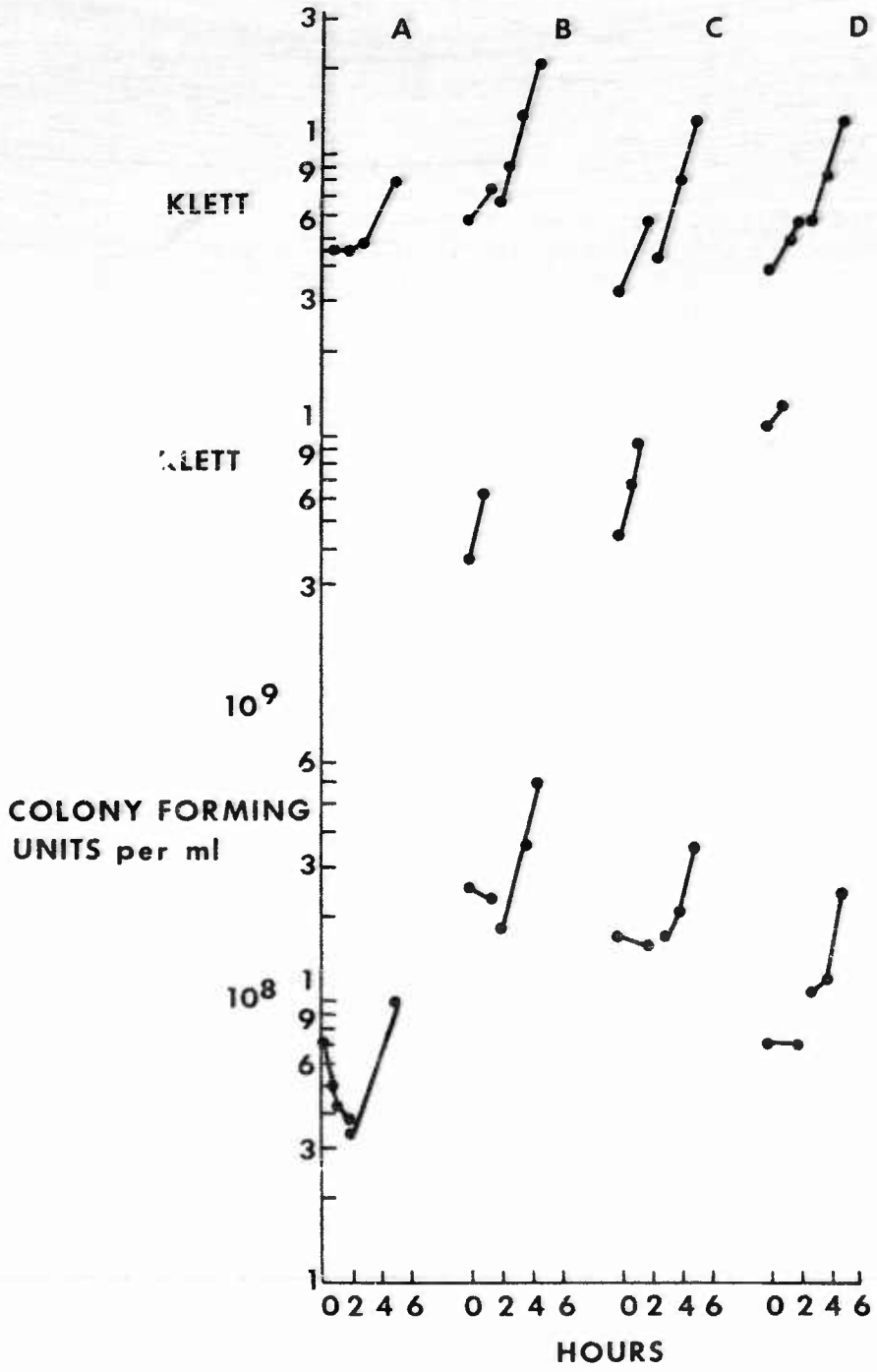

Figure 7. Growth of B. subtilis Strain W23S^r Methionine$^-$ under β-Phenethyl Alcohol Inhibition and Recovery Conditions.

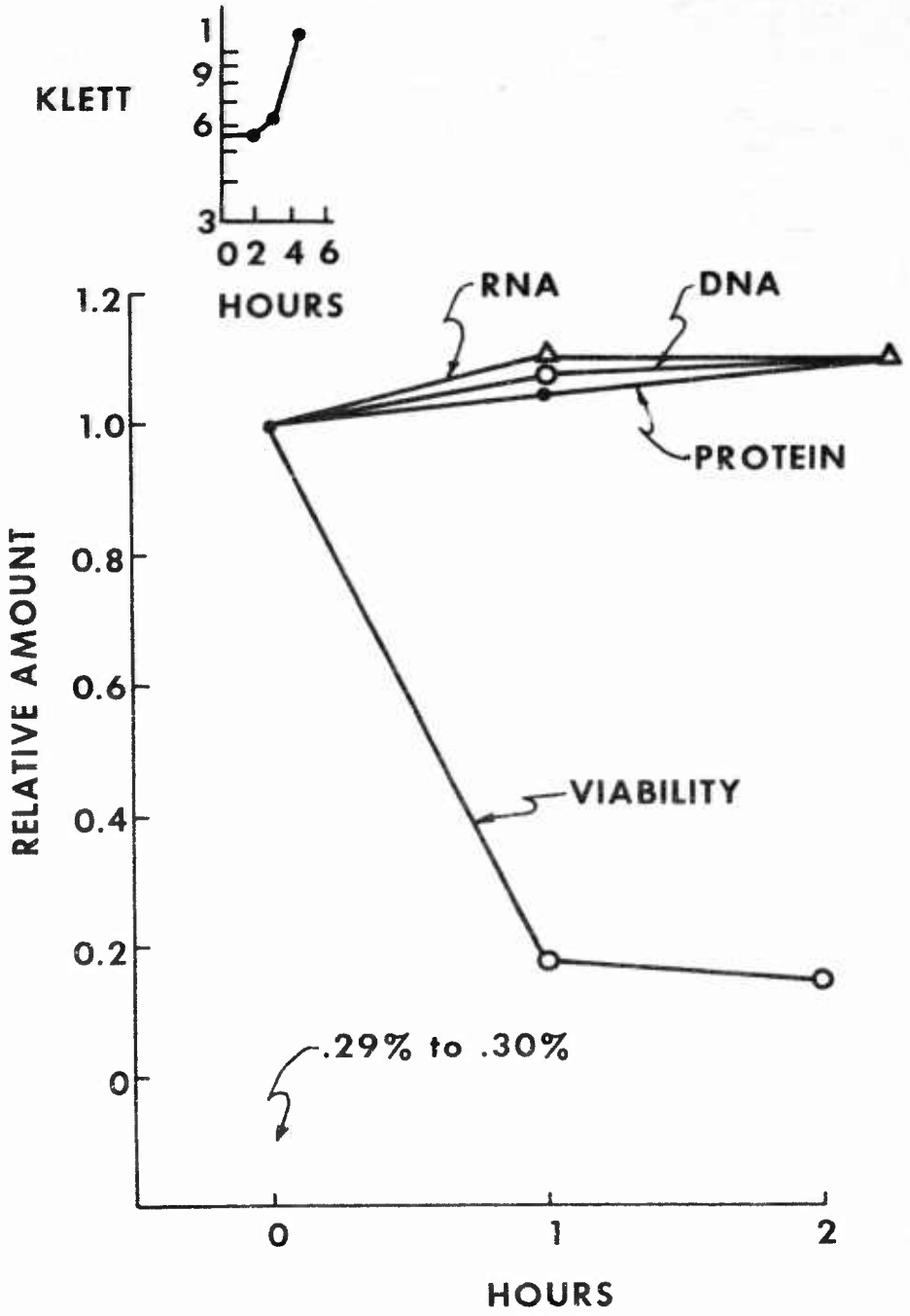

Figure 8. B. subtilis Strain W23S^{r} Methionine^{-} under β-Phenethyl Alcohol Inhibition.

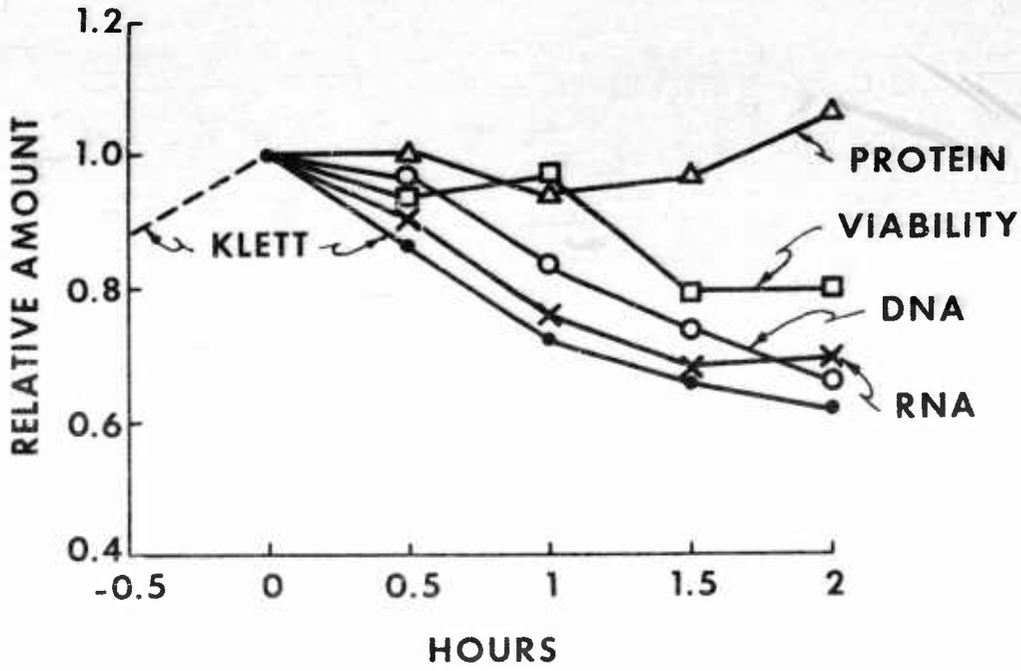

Figure 9. B. subtilis Strain W23S^{r} Methionine^{-} under β-Phenethyl Alcohol Inhibition (Log Phase Cells as Starting Material).

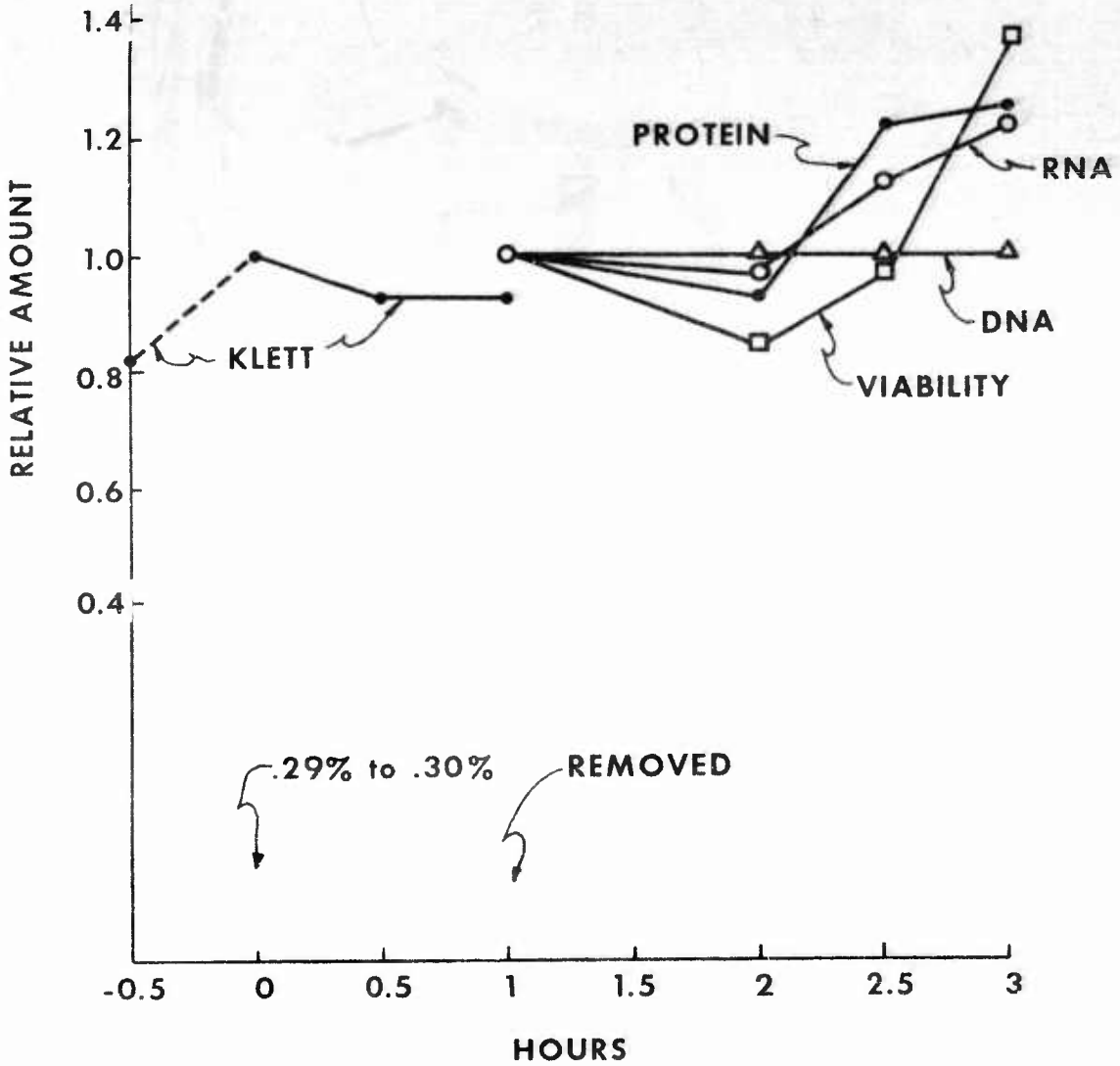

Figure 10. B. subtilis Strain W23S^{r} Methionine^{-} under β-Phenethyl Alcohol Inhibition and Recovery.

Finally, we have examined synthesis in recovery cells as shown in Fig. 10. Late log-phase cells (equivalent to those shown in column D of Fig. 7) were suspended in PEA at time 0. After one hour PEA was removed. The cells were resuspended in fresh growth medium and returned to 37 C. Fig. 10 indicates that under these conditions no synthesis takes place for one hour. RNA and protein synthesis then begin. DNA synthesis was not found to resume during the period examined in this experiment.

IV. DISCUSSION

PEA inhibition of synthesis in B. subtilis could be a useful tool for investigating numerous parameters in chromosome replication. The studies reported here are aimed at obtaining a working knowledge of PEA inhibition in B. subtilis. The first experiments presented show that the critical inhibitory concentration of PEA falls within very narrow limits. Repeated attempts to find a PEA concentration that would prevent growth of cells of strain 168 without causing permanent loss of viability have failed when stationary cells are used as inoculum. On the other hand, 0.28% (v/v) PEA proved satisfactory to inhibit log-phase cells of this strain with little loss of viability. Unfortunately, it was impossible to reverse the effects of inhibition by removal of the inhibitor.

In contrast, strain W23 was capable of recovering from PEA inhibition. Fig. 7 shows that cells in different phases of the growth cycle respond differently to PEA inhibition and relief from PEA inhibition. If stationary-phase cells are challenged, part of the population is inactivated in PEA. However, the survivors are able to recover and achieve log-phase growth. Cells taken from mid-log phase are prevented from dividing when in PEA but are not inactivated. These cells recover rapidly when removed from PEA and achieve maximal rates of growth. Late-log-phase cells are similar to mid-log-phase cells in their ability to survive in PEA. When removed from PEA these cells do not resume synthesis as rapidly as those in mid-log phase. Furthermore, they do not achieve as rapid a growth rate as mid-log-phase cells do upon recovery.

Comparable findings are reflected in the results of the chemical studies reported. Stationary-phase cells were found to stop net synthesis of DNA, RNA, and proteins when confronted with PEA. Log-phase cells similarly stop synthesis in PEA. In addition they appear to respond to the inhibitor more thoroughly and rapidly. During the first hour of recovery from inhibition, no RNA or protein synthesis was detected. No DNA synthesis was detected in the 2-hr period examined.

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This pattern of initiation of synthesis is qualitatively similar to the pattern of synthesis found in germinating spores of B. subtilis.[7] Isotopic studies are currently underway to examine these similarities.

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